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APPLICATION

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TITLE: **METHOD FOR IDENTIFYING MICROBIAL PROLIFERATION GENES**

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METHOD FOR IDENTIFYING MICROBIAL PROLIFERATION GENES

Statement as to Federally Sponsored Research

Research reported herein was supported in part by the National Science Foundation and the National Institutes of Health. The United States government may have certain rights in this invention.

Background of the Invention

Genes that encode proteins essential for microbial growth or viability are useful targets for antibiotics because inhibition of such proteins by an antibiotic can reduce or eliminate the spread of infectious disease in a patient. The emergence of bacteria resistant to multiple antibiotics has led to renewed interest in isolating variants of known antibiotics and also in identifying new required genes and the corresponding gene products that could serve as new targets for novel antibiotics.

Approximately fifteen different bacterial proteins encoded by essential genes have been used as targets for antibiotics. Such target proteins include ribosomal proteins, gyrase, RNA polymerase and proteins involved in the synthesis of the peptidoglycan layer and its precursors. However, there are estimated to be more than 4000 putative genes in the genome of the bacterium *Escherichia coli* and it is not known how many of these genes are required for growth and/or viability. The discovery of additional required genes could facilitate the search for new

antibiotics.

Microbial genes required or essential for growth and/or viability have been identified by two major techniques. One approach is to determine the nucleotide sequence of the genome of a microbial species of interest. Sequence information is then compared to sequences in computer databases to identify possible functions for the putative gene sequences. To prove that a putative gene identified only by sequence comparisons is in fact an essential gene, however, a null or "knockout" mutation must be made in the gene. It must be shown that a microorganism containing the null mutation cannot survive unless it is complemented by a wild type allele of the gene. Such an approach is time consuming and, in some species, can be difficult.

A second approach is to isolate conditionally lethal mutants by means such as chemical mutagenesis. A common type of conditional lethal mutation is a temperature-sensitive mutation, in which the mutant is non-viable at higher temperature such as 42° C but is viable at a lower temperature such as 30° C. The mutation is then mapped and identified by cloning and genetic complementation. The mutagenesis techniques employed are sometimes not completely random, thus reducing the likelihood of identifying a required gene that does not have a "hot spot" for the mutagen used. Moreover, products of some required genes may not be amenable to the formation of conditional lethal mutants. Such essential genes would not be identified by this approach.

Summary of the Invention

The present invention is based on the discovery that microbial genes and their encoded proteins essential for growth and/or viability can be identified by introduction into a

microorganism of an exogenous nucleic acid having sequence similarity to the microbial gene. The exogenous nucleic acid can be, for example, an antisense RNA that is derived from a chromosomal fragment cloned downstream of an inducible promoter. The endogenous gene can be identified as required by the inhibition of proliferation of microorganisms containing or expressing the exogenous nucleic acid, compared to the proliferation of microorganisms that do not contain or are not expressing the nucleic acid. The invention facilitates the rapid, efficient identification of microbial genes not previously known to be required for growth or viability.

It will be evident to those skilled in the art that the invention can be applied to any organism for which it is feasible to 1) isolate and fragment the chromosome of the organism, 2) re-introduce exogenous nucleic acid fragments of the organism or fragments substantially similar to those of the organism back into the organism and 3) culture the organism without the exogenously added nucleic acids.

The present invention advantageously permits the rapid, inexpensive identification of microbial genes that heretofore have not been identified as required for survival. The method comprises the steps of introducing an exogenous nucleic acid into a microorganism and determining the proliferation of the microorganism relative to the proliferation of the microorganism when the exogenous nucleic acid is not present or not expressed. The exogenous nucleic acid has substantial sequence identity to an endogenous microbial gene. The exogenous nucleic acid can be operably linked to a regulatory element effective for controlling expression of the nucleic acid, either in sense orientation or in antisense orientation to the regulatory element. The regulatory element can control expression via chemical induction of the exogenous nucleic acid. The microorganism can be, for example, a bacterium, either gram-negative or gram-

positive, a fungus, or an Archaebacteria.

The novel genes and the proteins encoded by such genes are promising targets for new antimicrobial agents. A method for identifying such agents comprises the steps of contacting an agent with a microorganism having a proliferation gene and determining the effect of the agent on the proliferation gene or a product of the proliferation gene. The proliferation gene can be endogenous to the microorganism, or can be from another source microorganism. The effect of the agent on the proliferation gene can be, for example, on transcription or on translation of the proliferation gene.

Specific genes and their mRNAs identified by the novel method can be targets for antisense polynucleotide inhibitors, and these antisense polynucleotides can have therapeutic value. A method for inhibiting expression of a microbial proliferation gene comprises contacting a microorganism having a proliferation gene with an antisense polynucleotide having substantial sequence identity to the proliferation gene.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

Figure 1 shows the nucleotide sequence of an antisense RNA to *E. coli lepB* mRNA,

expressed from the DNA insert in plasmid pJB37.

Figure 2 shows the nucleotide sequence of an antisense RNA to *E. coli viaA* mRNA, expressed from the DNA insert in plasmid pJB53.

Figure 3 shows the nucleotide sequence of a 714 nucleotide *Pst*I-*Hin*dIII fragment of the *E. coli viaA* gene cloned in pJB53.

Figure 4 shows the nucleotide sequence of an antisense RNA to *E. coli ddlB* mRNA, expressed from the DNA insert in plasmid pJB57.

Figure 5 shows the nucleotide sequence of an antisense RNA to *orf1* of the *E. coli ampG* operon, expressed from the DNA insert in plasmid pJB59.

Figure 6 is a bar graph showing the effect of inducing *lepB*, *ddlB*, *viaA* or *orf1* antisense RNA expression on colony formation in *E. coli* DH5α. Striped bars indicate colony forming units (CFUs) per ml of culture for strains grown on semi-solid media in the absence of the inducer IPTG. Solid bars indicate CFUs/ml for strains grown in the presence of IPTG.

Figure 7 is a graph showing growth curves of *E. coli* DH5α containing pJB37, pJB57 and pJB59 in liquid media in the presence and absence of IPTG.

Figure 8 is a graph showing the viability of DH5α strains at various times after induction of antisense RNA expression. Strains contained the following plasmids: squares: pJB37; diamonds: pJB53; circles: pJB57; and triangles; pJB59.

Figure 9 is a graph showing the effect of varying concentrations of inducer on the viability of DH5α containing pJB37 (panel A) or pJB53 (panel B).

Figure 10 is a bar graph comparing the effect of ampicillin or spectinomycin selection on viability of DH5α strains expressing *lepB*, *ddlB*, or *orf1* antisense RNAs. Ampicillin selection

(narrow stripes), ampicillin selection with 1 mM IPTG (gray), spectinomycin selection (wide stripes) and spectinomycin selection with 1 mM IPTG (solid).

Figure 11 shows the nucleotide sequence of an 836 nucleotide *Pst*I/*Hin*dIII fragment of the *E. coli secA* gene cloned in pJB3.

Figure 12 shows the nucleotide sequence of an antisense RNA complementary to *E.coli secA* mRNA.

Description of the Preferred Embodiments

The present invention provides an efficient strategy for identifying microbial proliferation genes. Proliferation genes are genes that are necessary for proliferation, *i.e.*, in the absence of gene transcript and/or gene product, growth or viability of the microorganism is reduced or eliminated.

Microorganisms can be used in the invention as a pure culture or may be used as a mixed culture, *i.e.*, more than one strain or species may be used in the novel method. Microorganisms suitable for use in the method include both prokaryotes and eukaryotes. Bacterial species suitable for use in the method include substantially all bacterial species, both animal and plant pathogenic species, as well as non-pathogenic species. Preferred species are pathogens of mammals, especially pathogens of humans such as *Staphylococcus aureus*.

Either gram-negative and gram-positive bacterial species can be used, e.g., gram-positive *Enterococcus*, *Bacteriodes*, *Clostridium* and *Staphylococcus* species and gram-negative *Escherichia*, *Pseudomonas*, *Haemophilus*, *Enterobacter*, *Vibrio*, *Salmonella*, *Helicobacter* and *Moraxella* species. *Mycobacterium* species such as *Mycobacterium tuberculosis* are also suitable

for use in the method disclosed herein. Eukaryotic microbes suitable for use in the method include, without limitation, *Cryptococcus neoformans*, *Nocardia spp.*, *Coccidiodes immitis*, *Toxoplasma spp.*, *Leishmania spp.*, *Pneumocystis*, *Trypanosoma spp.*, *Giardia spp.*, *Plasmodia spp.* (e.g., *Plasmodium falciparium*), *Histoplasma capsulatum*, and *Candida albicans*.

The novel method for identifying microbial proliferation genes comprises the step of introducing an exogenous nucleic acid into a microorganism. The exogenous nucleic acid may be obtained, for example, from a restriction digest of total genomic (*e.g.*, chromosomal and episomal) DNA from a microorganism of interest, or from total genomic DNA that has been subjected to physical shear. Sheared DNA may result in a more random set of fragments than would otherwise be generated by restriction digestion. Nucleic acid fragments obtained by partial or total restriction digestion or by shearing can be size selected by agarose gel electrophoresis or sucrose gradients, if desired. Exogenous nucleic acid can also be obtained by chemical synthesis (synthetic DNA having a random sequence or synthetic DNA based on information in a nucleic acid sequence database), from a cDNA library, or by other means known to the art.

An exogenous nucleic acid of the present invention can be in the form of RNA or in the form of DNA, including cDNA, synthetic DNA or genomic DNA. The DNA can be double-stranded or single-stranded and, if single-stranded, can be either a coding strand or non-coding strand. RNA can be, for example, mRNA or a combination of ribo- and deoxyribonucleotides.

An exogenous nucleic acid of the present invention has substantial sequence identity to an endogenous microbial proliferation gene. It should be appreciated, however, that it is not necessary to have prior knowledge of sequence identity to an endogenous gene. In fact, an

advantage of the present method is that a plurality of randomly generated nucleic acid fragments, whose identity to particular endogenous genes has not been determined, can be screened to identify a fragment (or fragments) that inhibit microbial proliferation. The relationship between such a fragment and the endogenous microbial gene can then be determined.

An exogenous nucleic acid has at least about 70% sequence identity, preferably at least about 80% sequence identity, more preferably at least about 90% sequence identity to the endogenous microbial proliferation gene. Sequence identity can be determined, for example, by computer programs designed to perform single and multiple sequence alignments. Nucleic acids having at least about 70% nucleotide sequence identity to the endogenous gene can also be identified by their ability to hybridize to the endogenous gene under conditions of moderate stringency. Nucleic acids having at least about 80% sequence identity, or at least about 90% sequence identity, or at least about 95% sequence identity to the endogenous gene can hybridize under high stringency conditions.

Hybridization can be measured by Southern analysis (Southern blotting), a method by which the presence of DNA sequences in a target nucleic acid mixture are identified by hybridization to a labeled oligonucleotide or DNA fragment probe. Southern analysis typically involves electrophoretic separation of DNA digests on agarose gels, denaturation of the DNA after electrophoretic separation, and transfer of the DNA to nitrocellulose, nylon, or another suitable membrane support for analysis with a radiolabeled, biotinylated, or enzyme-labeled nucleic acid as described in sections 9.37-9.52 of Sambrook *et al.*, *MOLECULAR CLONING: A Laboratory Manual*, Vol. 2, Chapter 11, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).

An exogenous nucleic acid can hybridize to the endogenous gene under moderate stringency conditions or under high stringency conditions. High stringency conditions are used to identify nucleic acids that have a high degree of homology or sequence identity to one another. High stringency conditions can include the use of a denaturing agent such as formamide during hybridization, *e.g.*, 50% formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, and 75 mM sodium citrate at 42°C. Another example is the use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS. Alternatively, low ionic strength and high temperature can be employed for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate (0.1X SSC); 0.1% sodium lauryl sulfate (SDS) at 65°C.

Moderate stringency conditions are hybridization conditions used to identify nucleic acids that have less homology or identity to one another than do nucleic acids identified under high stringency conditions. Moderate stringency conditions can include the use of higher ionic strength and/or lower temperatures for washing of the hybridization membrane, compared to the ionic strength and temperatures used for high stringency hybridization. For example, a wash solution comprising 0.060 M NaCl/0.0060 M sodium citrate (4X SSC) and 0.1% sodium lauryl sulfate (SDS) can be used at 50°C, with a last wash in 1X SSC, at 65°C. Alternatively, a hybridization wash in 1X SSC at 37°C can be used.

Hybridization can also be done by Northern analysis (Northern blotting), a method used to identify RNAs that hybridize to the nucleic acid used as the probe. The probe is labeled with a

radioisotope such as ^{32}P, by biotinylation, or with an enzyme. The RNA to be analyzed can be electrophoretically separated on an agarose or polyacrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe, using standard techniques well known in the art such as those described in sections 7.39-7.52 of Sambrook *et al.*, *supra*.

The exogenous nucleic acid can be less than about 400 nucleotides in length, *e.g.*, from about 10 to about 400 nucleotides in length. An exogenous nucleic acid can also be greater than about 400 nucleotides in length, *e.g.*, from about 10 to about 5,000 nucleotides in length. It is generally preferred that an exogenous nucleic acid of about 15 to about 1,500 nucleotides in length be used in the method, e.g., at least about 15 nucleotides, or at least about 30 nucleotides, or at least about 100 nucleotides.

In some embodiments, the nucleotide sequence of the exogenous nucleic acid has substantial complementarity to the coding sequence of the endogenous microbial proliferation gene, as in an antisense RNA. In other embodiments, the nucleotide sequence of the exogenous nucleic acid is the same as or substantially similar to the coding strand sequence of the endogenous gene, *e.g.*, is a sense RNA. An exogenous nucleic acid preferably exerts a microbiostatic (e.g., bacteriostatic or fungistatic) or microbiocidal (e.g., bacteriocidal or fungicidal) effect by antisense inhibition of a microbial proliferation gene. For example, an exogenous nucleic acid can be DNA that is transcribed into an RNA molecule complementary (antisense) to mRNA produced by the endogenous microbial gene. In such an embodiment, the exogenous nucleic acid is the DNA. The expressed antisense RNA has the capability to inhibit translation of the mRNA and thereby inhibit the proliferation of the microbial cells.

There are several possible modes of action for antisense molecules. For example, the

Shine Dalgarno sequence and start codon of a bacterial proliferation gene mRNA could be targeted by an antisense nucleic acid, and thereby inhibit ribosomal loading onto the mRNA. Alternatively, a hybrid molecule could be formed between an antisense nucleic acid and a portion of the coding region of the microbial proliferation gene mRNA. In this case, the mechanism of antisense inhibition could be premature translational termination, due to inhibition of ribosome movement or decreased mRNA stability. As another alternative, a hybrid could be formed between an antisense nucleic acid and a portion of the microbial proliferation gene mRNA near the transcriptional terminator. In addition, a triple helix could form between the proliferation gene and the exogenous nucleic acid, thereby inhibiting transcription of the proliferation gene. In some instances, it is possible that more than one of the above mechanisms, or a different mechanism, may be responsible for the effect on proliferation. The present invention encompasses these and other mechanisms.

Alternatively, an exogenous nucleic acid exerts a microbiostatic or microbiocidal effect by sense inhibition of a microbial proliferation gene, *i.e.*, dominant negative suppression. For example, an exogenous nucleic acid can be DNA that is transcribed into an RNA molecule having a sequence substantially identical to mRNA produced by the endogenous microbial gene. The expressed sense RNA has the capability to inhibit the proliferation of the microbial cells, possibly by producing proteins or truncated peptides that interfere with normal function of the endogenous proliferation gene product.

As discussed herein, it is preferable to obtain a plurality of exogenous nucleic acids, *e.g.*, a library of restriction fragments of a microbial genome. Such a population of exogenous nucleic acids has substantial sequence identity to a large number of endogenous microbial genes,

preferably the entire genome. The population is screened in parallel for an effect on microbial proliferation; those fragments exerting an inhibitory effect on growth and/or viability are selected for further study.

Typically, an exogenous nucleic acid is introduced into a microorganism on a vector, *e.g.*, a plasmid or phage vector. Illustrative examples of vectors are the pLEX series of plasmids described in Diederich, L. *et al.*, Biotechniques 16:916-923 (1994). Other suitable vectors are known in the art. If desired, assays can be used to confirm that the exogenous nucleic acid has been effectively introduced in order to confirm that the antiproliferative effect is due to the exogenous nucleic acid and not due to some other factor.

Techniques are known in the art for the introduction of exogenous nucleic acid into microorganisms, e.g., gram-negative as well as gram-positive bacteria. Techniques for introducing exogenous nucleic acid include, without limitation, lambda phage *in vitro* packaging, freeze-thaw mediated transformation, $CaCl_2$-mediated transformation, liposomes, electroporation, natural transformation, conjugation and particle gun transformation.

An exogenous nucleic acid preferably is operably linked to at least one suitable regulatory sequence to facilitate expression of the exogenous nucleic acid. Regulatory sequences typically do not themselves code for a gene product. Instead, regulatory sequences control the expression level of the exogenous nucleic acid. An exogenous nucleic acid can be operably linked to a suitable inducible regulatory sequence in antisense orientation or in sense orientation.

Examples of regulatory sequences are known in the art and include, without limitation, promoters induced in response to chemical inducers, promoters induced in response to environmental changes (temperature shifts, radiation and the like) and promoters repressed in

response to the presence of chemical agents. Examples of promoters induced in the presence of chemical inducers include the *E. coli lac* promoter (induced with isopropyl-β-D-thiogalactopyranoside, also known as IPTG), the *tet* promoter (induced with tetracycline) and the hybrid *tac* promoter (induced with IPTG). Examples of promoters repressed in response to a chemical repressor include the *trp* promoter (repressed by tryptophan, a corepressor). Other suitable regulatory sequences may be included if desired. For example, the *rrnB* transcriptional terminator 3' non-coding region, *rrnBt1t2* may be used.

Molecular biology techniques for linking an exogenous nucleic acid to a regulatory sequence effective for controlling expression of the nucleic acid are known in the art. In some embodiments, more than one regulatory element is operably linked to the exogenous nucleic acid, provided that the nucleic acid can be expressed when desired.

The novel method also includes the step of determining the effect of the exogenous nucleic acid on microbial proliferation, relative to the effect in the absence of the exogenous nucleic acid. For example, the exogenous nucleic acid can be operably linked to an inducible promoter and the effect on proliferation can be measured after induction of expression. The effect on proliferation is compared to microbial proliferation when the nucleic acid is not induced and expressed. Means for determining the effect on proliferation include, for example, measuring colony-forming units (CFU), measuring light scattering (optical density), measuring the number of microorganisms in a particle counter, measuring respiratory activity, measuring fluorescence of cell cultures or individual cells after addition of fluorescent dyes and measuring incorporation of precursors to macromolecular (RNA, DNA, protein, cell wall) synthesis, measuring uptake of metabolites and measuring transport.

Typically, microbial cells are cultured for a period of time to allow the exogenous nucleic acid to inhibit endogenous gene expression. Culturing may occur on solid media or in liquid media, on complex media or minimal media. Culturing may be carried out during an exponential growth phase, stationary growth phase, or during a stress phase. Nevertheless, it should be appreciated that the effect on proliferation can be determined without culturing the microorganism. For example, inhibition of a proliferation gene can be identified by a rapid effect on respiratory activity, a measurement that can be carried out under conditions that do not support long-term growth and viability. Techniques for determining the effect on proliferation that do not involve culturing of microorganisms are within the scope of the present invention.

Inhibition of bacterial growth is readily determined by comparative or replica plating. Bacteria are plated on solid media under conditions in which there is no production or expression of exogenous nucleic acid. Bacterial colonies are subsequently replica plated to a second plate of solid media under conditions in which production or expression of exogenous nucleic acid occurs. Clones that cannot grow in the presence of exogenous nucleic acid are chosen from the first plate for identification.

Culturing may occur for a period of time appropriate for the strain or species being tested. For example, about a 3 hour delay may occur before growth inhibition is observable with an antisense mechanism. Expressed antisense RNA must bind to and inhibit translation of its chromosomally encoded mRNA counterpart. Reduction in protein gene product may be detectable after 1 hour, but even if translational inhibition were 100% it may take several generations to "dilute out" the active gene product below levels required for viability.

It is often preferable to measure the effect on proliferation under culture conditions

similar to those in which it is desired to determine the requirement for the endogenous gene. For example, proliferation genes in a microbial human pathogen are preferably identified under conditions that relate to those found when the pathogen has infected a human, because genes required under such conditions are generally of more interest. It can be useful in some instances to identify proliferation genes under more than one set of conditions, *i.e.*, to determine if the gene is conditionally required. However, the most useful microbial proliferation genes are those genes necessary for growth and/or viability under substantially all conditions, because protein products of such genes are more desirable as targets for novel antibiotics.

Proliferation genes are often highly conserved among different species, for example, different bacterial species. Thus, microbial genes that have been identified as proliferation genes in one species or strain by the method disclosed herein can be used to search for related genes in other species or strains.

In some embodiments, an exogenous nucleic acid is not introduced on a vector (*e.g.*, a plasmid or phage vector), but is introduced as a non-replicating polynucleotide in an amount sufficient to exert an effect on the corresponding endogenous gene without the need for amplification of the amount *in vivo*. Such exogenous nucleic acids typically are antisense polynucleotides, including both short RNA oligonucleotides, typically 10-50 nucleotides in length, as well as longer RNA fragments that may exceed the length of the endogenous microbial gene itself. Antisense nucleic acids useful for the present invention are substantially complementary to regions of the corresponding proliferation gene mRNA. Hybridization of antisense nucleic acids to their target transcripts can be highly specific as a result of complementary base pairing. The capability of antisense nucleic acids to hybridize is affected by

such parameters as length, chemical modification and secondary structure of the transcript which can influence nucleic acid access to the target site. An antisense polynucleotide can also be a DNA segment that synthesizes an antisense RNA inside the cell.

In selecting the preferred length for a given nucleic acid, a balance must be struck to gain the most favorable characteristics. Shorter nucleic acids such as 10-to 15-mers, while offering higher cell penetration, have lower gene specificity. In contrast, while longer nucleic acids of 20-30 bases offer better specificity, they show decreased uptake kinetics into cells. See Stein *et al.*, "PHOSPHOROTHIOATE OLIGODEOXYNUCLEOTIDE ANALOGUES" in Oligodeoxynucleotides - Antisense Inhibitors of Gene Expression, Cohen, Ed. McMillan Press, London (1988).

The present invention encompasses both oligomeric nucleic acid moieties of the type found in nature, such as the deoxyribonucleotide and ribonucleotide structures of DNA and RNA. The invention also encompasses synthetic, modified analogues that are capable of binding to an endogenous microbial gene or its mRNA.

In some embodiments, therefore, an antisense polynucleotide of the present invention is a phosphorothioate or methyl phosphonate-linked analogue, which can be nuclease-resistant and thus have increased stability. Alternatively, an antisense polynucleotide can be a peptide nucleic acid, which can be nuclease-resistant and have high affinity for a complementary strand. See, e.g., U.S. Patents 5,539,082 and 5,539,083. Nucleic acids can also have adducts that permit cross-linking between strands of an antisense or sense hybrid. Persons of ordinary skill in this art will be able to select other linkages for use in the invention. These modifications also may be designed to improve the cellular uptake and stability of the nucleic acids.

Phosphorothioate modified DNA nucleic acids typically are synthesized on automated DNA synthesizers available from a variety of manufacturers, *e.g.*, available from Perkin-Elmer/Applied Biosystems (Foster City, CA). These instruments are capable of synthesizing nanomole amounts of nucleic acids as long as 100 nucleotides. Shorter nucleic acids synthesized by modern instruments are often suitable for use without further purification. If necessary, nucleic acids may be purified by polyacrylamide gel electrophoresis or reverse phase chromatography. See Sambrook *et al.*, MOLECULAR CLONING: A Laboratory Manual, Vol. 2, Chapter 11, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).

Illustrative embodiments of antisense polynucleotides includes polynucleotides having substantial sequence complementarity to the *E.coli lepB*, *viaA*, *ddlB*, *orf1*, and *secA* mRNAs, as well as oligonucleotide subfragments thereof, e.g., about 10 to about 100 nucleotides in length, or about 10 to about 60 nucleotides in length, or about 20 to about 50 nucleotides in length. A desired antisense oligonucleotide can be readily identified by preparing and testing overlapping portions of, e.g., an RNA of Figure 1, 2, 4-5 or 12 for antiproliferative effects as described herein. For example, antisense oligonucleotides about 30 nucleotides in length, each having a 10 nucleotide overlap with adjacent oligonucleotides, can be synthesized and tested for antiproliferative effects. Those oligonucleotides having such an effect are useful as antiproliferative agents as described herein.

Once a microbial proliferation gene has been discovered, the invention provides a method for identifying an antimicrobial agent targeted at the novel gene or gene product. The method includes the step of contacting an agent to be tested for its antimicrobial properties with a microorganism having a proliferation gene. The proliferation gene present in the microorganism

may be a gene endogenous to the microorganism. Alternatively, the proliferation gene can be one that has been introduced from a second microorganism. For example, a proliferation gene from a species for which it is more difficult to evaluate an agent can be introduced into a species for which it is less difficult to evaluate an agent, in order to facilitate testing of various agents.

Contacting includes conditions which allow the test agent to exert an effect, if any, on microbial proliferation, and can occur either in solution or in solid phase. In preferred embodiments, a plurality of agents are tested, *e.g.*, a combinatorial library can be used for screening. The agent is determined to be an antimicrobial agent when the agent is found to exert an effect on a microbial proliferation gene, its mRNA, or its encoded protein as described herein.

The novel screening method is useful for identifying new classes of antimicrobial agents directed at the endogenous microbial proliferation protein targets identified as described herein. Agents suitable for use in the method include inorganic chemical compounds, organic chemical compounds, peptidomimetics, and biologic compounds such as peptides, polypeptides, oligonucleotides and polynucleotides. Typically, the method is useful for identifying an agent that is itself microbiostatic or microbiocidal, *e.g.*, is potentially useful as an antibiotic. In some instances, an agent is useful for identifying a class of structurally related agents that will have efficacious microbiostatic or microbiocidal properties.

If desired, indirect means may be used to determine the effect of an agent on microbial proliferation. For example, an agent can be tested for its effect on enzyme activity if the proliferation gene encodes or is suspected of encoding an enzyme. If a protein does not have an easily assayable enzymatic activity, immunoassays can be carried out with antibody raised against a polypeptide or peptide product of a microbial proliferation gene, in order to identify

effects on transcription and translation of the endogenous gene. Northern blots or *in vitro* transcription assays can be used to determine the effect of the agent on transcription of the microbial proliferation gene. Such assays can be carried out on viable cells, on crude cell lysates or even on purified preparations of the endogenous gene, mRNA or gene product. In addition, structural bioinformatics can be used to identify putative inhibitors and likely mechanisms by which an agent exerts its microbiostatic or microbiocidal effect.

Agents identified by the method can be further evaluated by any method usually applied to the determine the efficacy and pharmacological activity of an antimicrobial agent.

Once a gene has been identified as a proliferation gene, it can be further characterized. Some identified genes will have been previously known as proliferation genes. Others, although having been previously sequenced, will not have been identified as proliferation genes. Others, although having been previously sequenced, are tentatively identified as proliferation genes based on sequence homology to an proliferation gene from another organism. Still others will not have been previously identified or characterized and can be cloned, if desired, using known techniques such as polymerase chain reaction (PCR) technology. See, *e.g.*, U.S. Patent 4,683,195, and subsequent modifications of the procedure described therein. Such genes can also be sequenced by known techniques such as dideoxy sequencing; open reading frames (ORFs) and putative regulatory elements assigned to various regions of the sequenced gene. Homology searches of nucleic acid and protein databases, such as the GenBank, EMBL, and Swiss Prot databases, are useful for deducing possible biochemical and metabolic functions of genes identified as required by the method disclosed herein.

Illustrative examples of proliferation genes include, for example, genes having substantial

sequence identity to the *E. coli viaA* gene, the *E. coli secA* gene, the *E. coli orf1* gene, the *E. coli ddlB* gene and the *E. coli lepB* gene. Such genes can be identified as proliferation genes by sense or antisense inhibition.

In some cases, genes are identified as proliferation genes based on the effect observed with sense constructs. For example, genes having substantial sequence identity to the *E. coli ugpB* gene and a gene having homology to the *E. coli fimF* and *fimD* genes can be considered proliferation genes because bacterial growth and/or viability is reduced or eliminated when a sense construct to such genes is expressed. Bacterial inhibition by antisense constructs would not ordinarily be expected to occur for such genes. Nevertheless, it should be appreciated that sense constructs that result in bacterial inhibition can be useful to derive antiproliferative agents. For example, peptides useful as antibiotics can be obtained, based on peptides made by the sense construct. Alternatively, nucleic acids useful as antibiotics can obtained based on the RNA made by the sense construct.

All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Examples

The following examples use many techniques well-known and accessible to those skilled in the art. Enzymes are obtained from commercial sources and are used according to the vendors' recommendations or other variations known to the art. Abbreviations and nomenclature are employed as commonly used in professional journals such as those cited herein.

Example 1.

Generation of *E. coli* Chromosomal Library

Bacterial strains, plasmids and media. Luria Broth was made as previously described (Sambrook *et al.*, 1989). M9 media (Sambrook *et al.*, 1989) was supplemented with 0.5% casamino acids and 0.2% glucose. Antibiotics were used at the following concentrations in plates and broth: ampicillin, 100 μg/ml; spectinomycin, 100 μg/ml. Isopropyl-β-D-thiogalacto-pyranoside (IPTG) was used at 100 μM or at 1 mM as indicated.

Isolation and cloning of exogenous nucleic acid. *E. coli* chromosomal DNA was digested with either *Pst*I and *Hin*dIII or *Eco*RI and *Bam*HI and ligated into the vector pLEX5BA, cut with the same enzymes (Diederich *et al.,* Biotechniques 16:916-923 (1994)) . Plasmid pLEX5BA contains: I) a Bujard promoter that has binding sites for lac repressor centered at -22 and +11 relative to the start of transcription, ii) a multiple cloning site downstream of the promoter, and iii) an *rrnBt1t2* transcriptional terminator after the multiple cloning site. Expression of fragments cloned downstream of the Bujard promoter can be induced with IPTG.

The double digestions were chosen to give fragments with a median length of 2 - 3 kb (Churchill *et al.*, 1990). The ligation mix was transformed into *E. coli* DH5α and transformants were selected on plates containing ampicillin. Colonies that grew on ampicillin were subsequently replica plated by physical transfer to a second ampicillin plate containing the inducing agent IPTG at a concentration of 100 μM. Colonies that did not grow in the presence of IPTG were chosen for further characterization.

Inhibition of bacterial growth after IPTG induction. Growth curves were carried out by back diluting cultures 1:20 into fresh media with or without 1 mM IPTG and measuring the

OD_{450} every 30 minutes (min). To study transcriptional induction in exponential phase, cultures were diluted $1:10^6$ or as indicated. The viability (CFU/ml) was determined over time by plating aliquots of the culture on agar with or without IPTG.

There were 53 clones whose proliferation was inhibited after IPTG induction, out of approximately 50,000 *Pst*I - *Hin*dIII clones screened. Out of 16,000 *Eco*RI - *Bam*HI cloned fragments, the growth of 15 transformants was inhibited after IPTG induction. Thus, approximately 0.1% of the inserts resulted in IPTG sensitivity when expressed from the Bujard promoter.

Plasmids pJB1 through pJB68 were cut with restriction enzymes to release the cloned inserts. Sizes ranged from 450 bp to 6 KB. Clones that had inhibition of colony formation after induction with 100 mM IPTG and had inserts less than 1050 bp were selected for further study. Certain plasmid derivatives and strains are listed in Table 1.

The nucleotide sequences of 11 inserts were determined, using plasmid DNA isolated by the method of Zyskind and Bernstein (1992). The primers used for sequencing the inserts were 5' TGTTTTATCAGACCGCTT 3' and 5' ACAATTTCACACAGCCTC 3'. These sequences flank the polylinker in pLEX5BA.

The sequences for the 11 chromosomal inserts were analyzed for similarity to sequences in GenBank using BLAST (Altschul, 1990). The presence of open reading frames and ribosome binding sites were scanned using the Genetics Computer Group programs FRAMES and CODONPREFERENCE, at default settings. Clones were designated as "antisense" if the cloned fragment was oriented to the promoter such that the RNA transcript produced appeared complementary to the mRNA from a chromosomal locus. Clones were designated as "sense" if

they coded for an RNA fragment that was identical to a portion of the wild type mRNA from a chromosomal locus. About 1/13,000 clones produced an inhibitory antisense RNA.

Table 1. E. coli K-12 strains (all F⁻) and plasmids

Strain	**Genotype/phenotype**
DH5α	supE44 Δ*lac*U169(ϕ80*lacZ*ΔM15) *hsd*R17 *recA1 endA1 gyrA96 thi-1 relA1*
JRG58201	Δ(*nadC-ampD-ampE-aroP*) *ampG*::*kan*
Plasmids	**Vector/Insert**
pLEX5BA	Ap^r cloning vector
pJB3	pLEX5BA with an 836 bp *Pst*I/*Hin*dIII fragment of *secA* in sense orientation
pJB37	pLEX5BA with a 546 bp *Pst*I/*Hin*dIII fragment of *lepB* in antisense orientation
pJB53	pLEX5BA with a 714 bp *Pst*I/*Hin*dIII fragment of *viaA* in antisense orientation
pJB57	pLEX5BA with a 1050 bp *Eco*RI/*Bam*HI fragment of *ddlB* in antisense orientation
pJB58	pLEX5BA with a 783 bp *Eco*RI/*Bam*HI fragment with homology to *fimF* and *fimD*
pJB59	pLEX5BA with a 451 bp *Eco*RI/*Bam*HI fragment of *orf1*/*ampG* in antisense orientation
pJB60	pLEX5BA with a 619 bp *Eco*RI/*Bam*HI fragment of *ugpB* in sense orientation
pCW37	pLEX5BA with a 546 bp *Pst*I/*Hin*dIII fragment of *lepB* in sense orientation
pCW53	pLEX5BA with a 714 bp *Pst*I/*Hin*dIII fragment of *viaA* in sense orientation
pCW57	pLEX5BA with a 1050 bp *Eco*RI/*Bam*HI fragment of *ddlB* in sense orientation
pCW59	pLEX5BA with a 451 bp *Eco*RI/*Bam*HI fragment of *orf1*/*ampG* in sense orientation
pAFΩ	Sp^r,St^r derivative of pLEX5BA containing 2 kb *Sma*I Ω fragment cloned into *Cla*I site
pAF37	pAFΩ with a 546 bp *Pst*I/*Hin*dIII fragment of *lepB* in antisense orientation
pAF53	pAFΩ with a 714 bp *Pst*RI/*Hin*dIII fragment of *ddlB* in antisense orientation
pAF57	pAFΩ with a 1050 bp *EcoRI*/*Bam*HI fragment of *viaA* in antisense orientation
pAF59	pAFΩ with a 451 bp *EcoRI*/*Bam*HI fragment of *orf1*/*ampG* in antisense orientation

Example 2

Identification of Proliferation Genes by Antisense Inhibition

Seven clones obtained as described in Example 1 inhibited bacterial proliferation and contained gene fragments in an antisense orientation.

Three of the clones contained fragments of the *lepB* gene; pJB12, pJB37 and pJB40. pJB37 contains a 546 bp *Hin*dIII - *Pst*I insert that expresses an RNA complementary to the carboxy terminal half of the *lepB* mRNA. The RNA sequence is shown in Figure 1. *LepB* encodes the leader peptidase responsible for proteolytic cleavage of the signal peptide from preproteins. Leader peptidase is required for viability and a temperature sensitive *lepB* mutant lyses at the nonpermissive temperature.

A fragment of an uncharacterized ORF was cloned twice in antisense orientation in pJB39 and pJB53. The antisense clone pJB53 contains a 714 bp *Pst*I - *Hin*dIII fragment of this putative ORF, designated herein as *viaA* (viability inhibited by antisense) and expresses an RNA that is complementary to the putative sense RNA. The sequence of the antisense RNA is shown in Figure 2 and the sequence of the DNA insert is shown in Figure 3. Plasmid pJB53 has been deposited with the American Type Culture Collection (ATCC), Rockville, Maryland as accession number _____.

One antisense clone, pJB57, contained a fragment of the *ddlB* gene. The D-alanyl:D-alanine ligase antisense clone, pJB57, contains a 1050 bp *Eco*RI -*Bam*HI fragment (with an internal *Eco* R1 site) that expresses an RNA antisense to the carboxy terminal portion of *murC* and the first 600 bases of *ddlB* including its start codon. The RNA sequence is shown in Figure 4. The product of the *ddlB* gene, the D-alanyl:D-alanine ligase, ligates 2 D-alanines together

which are destined to form part of the peptidoglycan layer. A temperature sensitive *ddlB* mutant could only grow for 40 min at the non-permissive temperature before lysing, presumably due to the compromised peptidoglycan layer. Lugtenberg E. and van Schijndel-van Dam, A., *J. Bact.* 113:96-104 (1973). There is a naturally occurring inhibitor of D-alanyl:D-alanine ligase activity, the antibiotic cycloserine.

The *ampG* operon antisense clone pJB59 contains a 451 bp *Eco*RI - *Bam*HI fragment which expresses an RNA antisense to a polycistronic mRNA containing *orf1* and *ampG*. The antisense RNA ends 64 nucleotides upstream of the *ampG* coding region. The *orf1* gene product has no known function but has been thought to be dispensable. *AmpG* forms a cytoplasmic membrane pore through which peptidoglycan subunits are translocated to signal inducible β-lactamase promoters when peptidoglycan damage occurs from β-lactam antibiotics. Mutations in *ampG* cause a deficiency in recycling of the peptidoglycan layer and lose 40% of their peptidoglycan per generation but remain viable. The sequence of the antisense RNA is shown in Figure 5.

Antisense RNAs to *lepB, ddlB, viaA* and *orf1* do not encode consensus ribosome loading sites. To determine if the chromosomal inserts within pJB37, pJB53, pJB57, and pJB59 could be translated into peptides, they were analyzed by computer for ribosome loading sites and open reading frames.

Plasmids pJB37, pJB53 and pJB57 do not have significant open reading frames or consensus ribosome loading sites relative to the inducible Bujard promoter. pJB59 does have an open reading frame relative to the Bujard promoter; however, no consensus ribosome loading site occurs in the antisense reading frames. The insert in pJB57 was shown to have a promoter which

was active in an *in vitro* transcription/translation assay.

The antisense inserts in pJB37, pJB53, pJB57 and pJB59 do not encode lethal peptides. Sense plasmids were constructed to examine whether the deleterious effects on cell growth and viability could be caused by peptides produced by internal promoters in the inserts present in pJB37, pJB53, pJB57 and pJB59.

Sense orientation plasmids were generated by cutting antisense plasmids pJB57 and pJB59 with the enzymes *Bst*BI and *Acc*I which generates compatible ends. Each fragment mixture was then religated in the presence of *Acc*I, which would recleave the plasmid if it religated in the "antisense" orientation but would not recognize the altered sites created by the fragment ligating in the "sense" orientation. Plasmids pCW57 and pCW59 contained the inserts from pJB57 and pJB59, respectively, in sense orientation.

To create pCW37 or pCW53, the inserts of pJB37 and pJB53 were amplified by PCR using the sequencing primers listed above. The PCR product was cut with *Hin*dIII and *Sma*I, filled in with Klenow and then cloned into the *Sma*I site of pLEX5BA. pCW37 and pCW53 contain the inserts from pJB37 and pJB53, respectively, in sense orientation. The orientation of all recombinants was verified by restriction analysis.

Cell proliferation for each strain was measured by diluting overnight cultures $1:10^6$ into M9 medium. After growth to an OD_{450} of 0.2, aliquots of each culture were plated on M9 agar without IPTG or M9 agar containing 1 mM IPTG. The results are shown in Figure 6. Sense plasmids pCW37, pCW53, pCW57, and pCW59 had no measurable effect on cell growth or colony formation when induced with IPTG. In contrast, cells carrying pJB37 or pJB53 were unable to form colonies when plated with IPTG. Colony formation by cells carrying pJB57 was

inhibited more than 100 fold. The colony formation of strains carrying pJB59 was inhibited more than 1000 fold. These results indicate that the inserts in pJB37, pJB53, pJB57 and pJB59 do not contain internal promoters expressing a lethal peptide in the sense orientation.

To test whether chromosomal inserts expressed proteins, 4 μg of CsCl purified plasmid templates was used in an *in vitro* transcription translation system for supercoiled templates (Promega, Madison, Wisconsin). The manufacturer's instructions were followed using 10 μCi ^{35}S-methionine and 100 μM IPTG in a 50 μl reaction. Samples were precipitated with acetone and electrophoresed on 15% SDS PAGE. Bands were detected by exposure to a Molecular Dynamics phosphorimager and to film. No detectable peptide product was made from clones pJB37 or pJB59.

Kinetics of inhibition during exponential growth by antisense RNA. The kinetics of antisense inhibition was examined for strains carrying pJB37, pJB53, pJB57 or pJB59. Overnight cultures of each strain were diluted 1:20 into LB media containing 1 mM IPTG or LB media without IPTG. The OD_{450} was then measured on the liquid cultures. Figure 7 shows the increase in OD_{450} during exponential growth for pJB37, pJB57 and pJB59, with and without induction of antisense RNA. As seen in Figure 7, transcriptional induction of antisense RNA did not have a significant effect on the increase in cell mass until about 3 hours after induction. Similar results were obtained for a strain carrying pJB53. After induction of antisense RNA expression, a few generations of cell division presumably are required in order to dilute endogenous gene product present at the initiation of induction. The cell mass for the strains expressing the *lepB* or *viaA* antisense RNAs (pJB37 and pJB53, respectively) began to decrease by about 4 hours, suggesting that cells had begun to lyse. Microscopic examination revealed that

cells expressing *lepB* antisense RNA did lyse, whereas cells expressing *viaA* antisense RNA did not. The cell mass for strains carrying *ddlB* (pJB57) and *orf1* (pJB59) stopped increasing after about 3 hours but did not decrease, suggesting that these cells did not lyse.

The OD_{450} of strains carrying the sense constructs described above continued to increase, both in the presence and in the absence of IPTG, showing that induction of the sense RNA had no effect on bacterial proliferation.

Inhibition of viability by antisense RNA. The viability over time of the four strains expressing the antisense RNAs described above was also examined. Overnight cultures of each strain were diluted 1:100 into LB medium containing 1 mM IPTG. Aliquots of each culture were plated on LB agar containing ampicillin at intervals thereafter to determine CFUs/ml.

Data for strains carrying plasmids pJB37, pJB53, pJB57 and pJB59 are shown in Figure 8. The data show that 6 hours after induction, viability is reduced by about 3 logs for strains expressing *lepB* or *viaA* antisense RNA. The data also show that growth is inhibited but viability is not reduced when *ddlB* or *orf1* antisense RNAs are expressed. Optical density for strains expressing *ddlB* or *orf1* antisense RNA increased over the period of the experiment, suggesting that cell size increases but cell division is inhibited for these strains. The results suggest that *lepB* or *viaA* antisense RNA is bacteriocidal, whereas *ddlB* or *orf1* antisense RNA is bacteriostatic.

Effect of different concentrations of transcriptional inducer. Experiments were carried out to determine the effect of different concentrations of the inducer IPTG on bacterial growth and viability with the two clones where antisense RNA is bacteriocidal. Overnight cultures were diluted 1:100 into LB media containing 0, 10, 100 500 or 1000 micro molar (μM)

IPTG. At the time of induction and every 2 hours thereafter, aliquots were plated on LB/ampicillin agar to determine CFUs/ml.

The results shown in Figure 9 indicate that the anti-proliferative effect of antisense to *lepB* or *viaA* is near maximal at 500 μm IPTG. Cell death occurred within 2 hours. A 2 fold increase to 1000 μm IPTG had little effect on the time of cell death. A 5 fold decrease in IPTG (100 μm) resulted in a longer period of survival (4 hours or more) before CFU began to decrease. However, cells did not survive overnight induction at 1000 μm IPTG, as shown in Figure 6. Very low levels of IPTG (10 μm) had minimal effect on colony forming ability.

***lepB* antisense induction inhibits leader peptidase activity.** The *lepB* gene encodes a leader peptidase that cleaves the leader sequence from preproteins such as maltose binding protein (MBP). Endogenous leader peptidase activity was examined in the presence of pJB37 antisense RNA, by measuring processing of pre-MBP (43.38 KD) to the mature 40.7 KD protein.

An overnight culture of pJB37/DH5α was diluted 1:100 in M9 media containing 0.2% casamino acids, 0.2% maltose, 100μg/ml ampicillin. The diluted culture was split into 2 flasks, one containing 1 mM IPTG and the other containing no IPTG. Samples were taken at the time of the split and at hourly intervals thereafter. Chloramphenicol was added to each sample to a final concentration of 150 μg/ml. Samples were placed on ice, centrifuged, washed with M9 salts, and precipitated with cold 10% trichloroacetic acid (TCA). After centrifugation, the TCA precipitated material was resuspended in buffer (0.125 M Tris-HCL, pH 6.8, 0.1 M NaOH, 1% w/v SDS, 0.1% Phenol Red). The amount of protein in each precipitate was normalized to the different optical densities of the samples by using 0.1 ml of buffer per ml of culture having an OD_{450} of 1.0. About 8 μl of each resuspended precipitate was mixed with an equal volume of 2X

cracking buffer (100 mM Tris-Cl pH6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and boiled for 10 minutes. Samples were electrophoresed on a 10% polyacrylamide-SDS gel as described in Sambrook *et al.*, *supra* (1989).

Gel-separated proteins were electrophoretically transferred to a Protran nitrocellulose membrane (Schleicher & Schuell) and the membrane incubated with rabbit anti-MBP antibody diluted to 1:10,000. After washing, the membrane was incubated with a 1:10,000 dilution of goat anti-rabbit antibody conjugated to horseradish peroxidase (BioRad). Proteins were detected with a chemiluminescence kit (DuPont-NEN) and captured on autoradiographic film. The image was scanned using a Molecular Dynamics densitometer. The results showed that unprocessed MBP is detectable after induction of *lepB* antisense transcription with IPTG. No unprocessed MBP is detectable in untreated cells. The data demonstrate that *lepB* antisense RNA results in inhibition of the endogenous bacterial gene product.

Lethality of pJB59 antisense RNA requires inhibition of *orf1*. The polycistronic mRNA that encodes *ampG* in *E. coli* has an upstream ORF, termed *orf1* herein. The antisense RNA made by pJB59 is complementary to *orf1*. In order to determine whether translational inhibition of *orf1* or of *ampG* was the cause of the lethality of pJB59 antisense RNA, pJB59 was transformed into the *ampG* null strain, JRG58201. Jacobs, C., *et al.*, EMBO J. 13:4684-4694 (1993). JRG58201 has a defect in the *ampG* gene but has no known defect in the *orf1* gene.

Growth and viability of JRG58201 containing pJB59 was inhibited after IPTG induction. Thus, the anti-proliferative effect of the antisense RNA of pJB59 requires at least the inhibition of *orf1* gene expression. Whether the anti-proliferative effect of the *ampG* gene product is also involved cannot be determined from this experiment.

Effect of different antibiotic selectable markers on antisense inhibition of growth.

The possibility that IPTG induction was sensitizing the cells to ampicillin was examined by transferring the inserts from plasmids pJB37, pJB53, pJB57 or pJB59 to the vector pAFΩ. Plasmid pAFΩ confers resistance to ampicillin, spectinomycin, and streptomycin. The resulting constructs were then tested for inhibition of bacterial growth using spectinomycin as the selectable marker rather than ampicillin.

pAFΩ was constructed by isolating the *Sma*I fragment containing Omega (Sp^r, Str^r) of pHP45. Prentki and Kirsch, Gene 29:303-313 (1984). The omega fragment was then ligated into the filled-in *Cla*I site of pLEX5BA.

To generate pAF37, pAF53, pAF57, and pAF59, the inserts of pJB37, pJB53, pJB57, pJB59, respectively, were amplified by PCR. The PCR products of pJB37 and pJB53 were cut with *Hin*dIII, filled in with Klenow, and cut with *Eco*RI before ligation into *Eco*RI-*Sma*I cut pAFΩ. The PCR products of pJB57 and pJB59 were cut with *Pst*I and *Eco*RI and ligated into identically cut pAFΩ. Ligation reactions were transformed into DH5α and ampicillin spectinomycin resistant transformants were selected. Generation of the correct constructs was verified by restriction analysis.

Overnight cultures of strains carrying plasmids pAF37, pAF57 or pAF59 were diluted 1:100 in LB medium containing spectinomycin. After growth to an OD_{450} of 0.2, cells were plated on ampicillin, ampicillin with 1 mM IPTG, spectinomycin, or spectinomycin with 1 mM IPTG, and CFUs/ml of culture were determined. The results are shown in Figure 10.

Figure 10 shows that induction of antisense nucleic acid under spectinomycin selection inhibits bacterial proliferation to the same extent as does antisense nucleic acid under ampicillin

selection. Similar results were obtained for pAF53. These results suggest that the inhibition of bacterial proliferation observed with these constructs is not due to an effect on ampicillin resistance.

In order to examine the effectiveness of the antisense RNAs in a related species, *Salmonella typhimurium* was transformed with pJB37 or pJB53. The ampicillin-resistant transformants were plated on LB with or without 1 mM IPTG. Growth was greatly inhibited after induction of the antisense RNA, suggesting that sufficient complementarity existed between the antisense RNAs and *S. typhimurium* genes to inhibit production of proliferation gene products in this species as well as in *E. coli*.

The data described above indicate that antisense clones inhibit cell growth by producing an antisense RNA. Transcription of the cloned fragment must occur because IPTG induction is necessary to inhibit proliferation. None of the inserts are lethal in a sense orientation. Thus, any peptide possibly made by the insert in pJB57 is not responsible for lethality. No detectable peptide product is made from the antisense RNA of clones pJB37 or pJB59 *in vitro*. The pJB37 (*lepB*) antisense RNA was shown to decrease leader peptide processing activity.

Example 3

Identification of Proliferation Genes by Sense Inhibition

Four of the clones described in Example 1 inhibited bacterial proliferation and contained gene fragments in sense orientation. Plasmid pJB3 contained a fragment of the *sec*A gene in sense orientation. Plasmid pJB60 contained a fragment of the *ugpB* gene in sense orientation. Plasmids pJB55 and pJB58 each contained a fragment in the sense orientation which expressed

portions of genes having sequence homology to the *E. coli fimF* and *fimD* genes (located at 34 min on the *E. coli* map).

The insert in the *secA* dominant lethal clone, pJB3, was identified as an 836 bp *Pst*I-*Hin*dIII fragment coding for *yacA* and the N-terminal 39 amino acids of the *secA* protein. The DNA sequence of the insert is shown in Figure 11. *SecA* is an ATP-dependent translocase of secreted proteins located at the cytoplasmic face of the membrane. *SecA* protein regulates its own translation by binding to a site overlapping the ribosome binding site, which causes dissociation of a preformed 30S-tRNA-fMet-mRNA ternary complex. Since this site is located on the cloned fragment in pJB3, the sense RNA produced by pJB3 after induction could be binding and potentially sequestering most of the *secA* protein, resulting in a fatal deficiency in trans-membrane transport. Alternatively, the truncated *secA* peptide produced by pJB3 could be poisoning the membrane complex or binding the wild type mRNA, down regulating translation of *secA*.

An antisense RNA complementary to *secA* mRNA would also be expected to inhibit bacterial proliferation. The nucleotide sequence of such an antisense RNA is shown in Figure 12.

The dominant lethal *ugpB* sense clone, pJB60, contains a 619 bp *Eco*RI - *Bam*HI fragment which encodes the amino-terminal portion of the *sn*-glycerol-3-phosphate binding protein.

The insert in the dominant lethal clone, pJB58, is a *Bam*HI - *Eco*RI fragment approximately 783 bp long. The plasmid insert contains two partial ORFs in a sense orientation. One partial ORF encodes the C-terminal portion of a protein which shows 59% similarity to

FimD protein. The other partial ORF encodes the N-terminal portion of a protein with 56% similarity to FimF protein.

To the extent not already indicated, it will be understood by those of ordinary skill in the art that any one of the various specific embodiments herein described and illustrated may be further modified to incorporate features shown in other of the specific embodiments.

The foregoing detailed description has been provided for a better understanding of the invention only and no unnecessary limitation should be understood therefrom as some modifications will be apparent to those skilled in the art without deviating from the spirit and scope of the appended claims.